again. Current models from single-virus microscopy suggest that fusion requires the engagement of several hemagglutinin trimers in close proximity. Our findings are in agreement with these models. If hemagglutinin trimers indeed shift closer together in the viral envelope upon cholesterol extraction, this would result in an increase in the rate of fusion.

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Cholesterol and Influenza Viral Fusion Mechanisms: Using Sterol Analogues to Probe Mechanism
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Fusion between the viral envelope and target cell membrane is a crucial step for infection of influenza and other enveloped viruses. The influenza envelope protein hemagglutinin provides a necessary driving force for fusion. However, both viral and target membrane composition can also have a significant impact on infectivity. Here, we examine the effect of sterol composition on viral membrane fusion kinetics.
We have previously shown that changes to cholesterol concentration in either synthetic target liposomes or viral membranes can alter both hemi-fusion and fusion kinetics. Increasing cholesterol in target liposomes caused a monotonic increase in fusion rate, while depleting viral cholesterol from the starting cholesterol:phospholipid ratio of 1:1 had a more complex effect. To further characterize what chemical properties of cholesterol are responsible for the observed changes and begin to dissect the responsible mechanism, we examined the effect of other related sterols. We tested seven cholesterol analogues, measuring fusion kinetics when each sterol was used in place of cholesterol in target liposomes or when cholesterol was extracted from the viral membrane and replaced by each sterol tested. As measured by fluorescence dequenching kinetics, fusion between X-31 influenza virus and target vesicles containing 20% sterol did not significantly change hemi-fusion or fusion rates among the sterols tested. Most sterols tested in the viral envelope had a minimal effect on fusion kinetics compared with cholesterol. However, replacing viral envelope cholesterol with cholesteryl sulfate significantly slowed both hemifusion and fusion. We are currently working to identify the chemical basis for this effect.

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Characterization of HIV-1 Entry Site Specificity using Single-Particle Tracking
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The first critical step in the HIV-1 infectious cycle is fusion of the viral membrane with the membrane of the host cell and penetration of the viral capsid into the host cytosol. Because the functionality of the HIV-1 fusion glycoprotein (Env) is pH-independent, it was thought that productive HIV-1 fusion occurs at the plasma membrane (PM). This hypothesis is supported by the observations that HIV-1 mediates fusion between adjacent cells and that cell-cell fusion occurs between Env and receptor/co-receptor expressing cells. To the contrary, it was recently demonstrated that, after engaging receptor/co-receptor at the cell surface, HIV-1 traffics via an endocytic pathway before fusing with the endosomal membrane. However, the previous virus labeling techniques were unable to reliably detect fusion with the PM. Here, we directly quantified the fraction of HIV-1 virions that fuse with the PM by co-labeling viral particles with a pH-sensor incorporated into the viral membrane and a content marker that is released into the cytoplasm upon fusion. In imaging viruses bound to living cells, virus fusion at neutral pH is manifested as loss of the viral content marker without change to the signal from the pH-sensor. Upon virus entry to an acidic compartment, the reference signal from the pH-sensor is completely quenched, thus precluding detection of subsequent fusion. We found that only a small fraction of fusion events occur at neutral pH, presumably at the cell surface or in early, pH-neutral vesicles. Our finding implies that the majority of HIV-1 virions enters and fuses after trafficking into acidic compartments. Notably, the viral pH sensor also revealed occasional recycling of HIV-1 particles to the cell surface followed by their re-internalization. This work was supported by NIH R01 GM054787.

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Recognition of Lipid Domain Boundaries by the HIV Fusion Peptide is an Essential Step for HIV Membrane Fusion
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"Lipid rafts" which are cholesterol-enriched regions of the plasma membrane have been recognized as possible platforms for HIV entry. However, due to their highly dynamic and nanoscopic nature in biological membranes, it is challenging to investigate the role of lipid rafts in HIV-host interactions. In this study, we have used model systems with microscopic raft-like domains (Lo phase) in supported lipid bilayers and giant unilamellar vesicles mimicking HIV envelopes and T-cell membranes. We show that the phase separation of HIV or T-cell lipid mixtures is cholesterol-dependent, and membrane binding and lipid mixing are much more efficient in vesicles with coexisting Ld and Lo phases than those with single Ld or Lo phase, indicating that lipid phase separation is necessary and sufficient for efficient membrane fusion. Interestingly, time-resolved TIRF microscopy demonstrates that the HIV fusion peptide preferentially targets Lo/Ld boundary regions and promotes membrane fusion at the interface between Lo and Ld phases. Analysis of individual fusion events shows that pure Ld phase vesicles proceed to hemifusion and only vesicles with Lo/Ld phase boundaries fuse fully. Based on our designed minimal systems for understanding lipid raft-dependent HIV-host interactions, we propose that recognition of domain boundaries by the HIV fusion peptide is an essential step for HIV entry.

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Post Fusion Structure of the Transmembrane Domain of the Ebola Virus Surface Glycoprotein
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Ebola virus (Ebov) is an enveloped virus causing hemorrhagic fever in humans and non-human primates with extremely high fatality rates. Fusion occurs when the virus reaches the endosomal compartment where the surface glycoprotein (GP) undergoes proteolytic cleavage and rearrangement. In this study we focus on the role of the transmembrane (TM) and membrane proximal (MPER) domains of Ebov GP in the fusion process. NMR spectroscopy was performed on a construct comprising Ebov TM and MPER (residues 632-676) to investigate its structure and role in fusion. Solution NMR studies show that the structure is composed of two helices. A dynamic N-terminal region (residues 632-642) is followed by a short MPER helix residing on the surface of the membrane (residues 643-651), a turn (residues 652-657), and the TM helix (residues 658-676). HSQC spectra of Ebov TM/MPER at pre- and post-fusion pH were similar suggesting a pH-independent role of this domain in fusion. Titration experiments revealed a binding site between the FL and TM domains at pH 5.5. The binding site is rich in aromatic residues on the TM (WTGW) and FL (YWTTQD) side. Liposome fusion assays showed that lipid mixing was enhanced when the liposomes contained Ebov TM/MPER. Taken together, we conclude that the Ebov TM/MPER and FL domains cooperate in fusion, but that only the FL structure [see refs. 1 and 2] responds to pH in this process.
1. Gregory et al. PNAS (2011) 108:11211-11216.
2. Gregory et al. J. Virol. (2014) 88:6636-6649.

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The Role of Acidic pH in Ebola Mediated Cell-Cell Fusion
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The low pH within endosomes promotes fusion of many viruses, including Ebola virus (EBOV) from within this compartment. We have developed a system that monitors aqueous and lipid dye mixing to measure fusion between cells that is mediated by the fusion protein, GP, of EBOV. Fusion is pH-dependent, and the extent of both aqueous and lipid dye mixing exhibits a maximum at pH 5.7. Some fusion occurs at neutral pH. Fusion requires cleavage of EBOV GP into two linked subunits. In a biological setting, cathepsins, whose activities are pH-dependent, are the responsible cleaving enzymes. Inhibiting cathepsins activity eliminated fusion. Introducing a recombinant cathepsin into the external solution largely restored pH-dependent fusion. In the laboratory, proper cleavage of EBOV GP can be achieved by using thermolysin and this treatment doubles the rate and extent of cell-cell fusion above that achieved by relying on endogenous cathepsins alone. For thermolysin-treated effector cells expressing EBOV GP, cathepsin inhibitors reduce the extent of fusion by roughly a factor of two. We have shown that EBOV GP mediated cell-cell fusion is reduced by proteinase K treatment, and have determined the stage of fusion at which EBOV GP becomes sensitive to this protease. We found that the EBOV GP fusion pore is initially small, and unlike other viral protein mediated fusion pores, the pore tends to remain small and typically does not enlarge. In addressing how acidic pH promotes EBOV GP mediated fusion, a mechanism which is somewhat controversial, we found that contributing equally are the increases in activity of cathepsins with lowered pH and the direct effects of low pH in promoting conformational changes of cleaved GP. Supported by NIH RO1 GM101539.